Identification and functional characterization of *Rhizobium leguminosarum* bv. viciae genetic systems involved in nickel homeostasis

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Summary

A collection of *Rhizobium leguminosarum* bv. viciae strains isolated from ultramafic and contaminated soils in Italy and Germany, respectively, was analyzed for resistance to nickel and cobalt ions. These assays led to the identification of strain UPM1137, which is able to grow at high concentrations of nickel and cobalt. In order to identify genetic systems involved in the homeostasis to these metals, a random mutagenesis was carried out in UPM1137 by inserting a Tn5-derivative minitransposon. As a result 4313 transconjugants were obtained, being 39 of them (0.90%) unable to grow at 1.5 mM $NiCl_2$. The identification of the transposon insertion site in these mutants showed that the disrupted genes encode proteins belonging to different functional categories, where the secreted and membrane proteins were the most numerous. The analysis of heavy metal resistance and phenotypes in symbiotic and free –living cells will define the contribution of these genes to metal homeostasis.

Introduction

Soils with high concentrations of heavy metals, either naturally or by contamination, are becoming more abundant. High metal concentration in soils affects fertility as well as microbial activity. Bacteria are able to develop several mechanisms to detoxify the cell and resist those stress conditions (Bruins et al., 2000). Analysis of bacteria isolated from ultramafic soils, or soils contaminated with heavy metals, might allow the identification of mechanisms required for survival in such inhospitable conditions.

Materials and Methods

The nitrogen fixation and nodulation phenotypes of strains UPM1131, UPM1132, UPM1133, UPM1134, UPM1135, UPM1136, UPM1137, UPM1138, UPM1139, UPM1140, UPM1141 and UPM1142 of *Rhizobium leguminosarum* bv. viciae (Fernández et al., 2005) were analyzed by inoculation of pre-germinated seeds of *Pisum sativum*. The analysis of nickel and cobalt resistance in strains of *R. leguminosarum* was performed in plates of TY medium supplemented with increasing concentrations of $NiCl_2$ and $CoCl_2$. For disk diffusion tests of metal resistance (Bauer et al., 1966), exponential cultures were inoculated in TY medium and disks soaked in 100 mM, 200 mM and 500 mM $NiCl_2$ or 20, 50 and 100mM $CoCl_2$, were placed on the plate. The diameter of the inhibition zone (mm) was measured after 48 hours of incubation.

Plasmid pSS240, containing the minitransposon Tn*5*SSoriR*gusA*, was introduced in strain UPM1137 by conjugation. Transconjugants affected on their growth on 1.5 mM $NiCl_2$ were selected, and the disrupted gene was identified by cloning in *Escherichia coli* and sequencing of the regions adjacent to the minitransposon with the aad1846 primer, complementary to vector pSS240. The sequences obtained were compared using BLAST program at the NCBI database. Additional information about genes and classification were obtained from the database RhizoBase (http://genome.kazusa.or.jp/rhizobase/).

Results and Discussion

A collection of *Rhizobium leguminosarum* bv. viciae strains isolated from soils of Italy and Germany containing high concentrations of heavy metals were analyzed for resistance to nickel and cobalt. Growth in TY medium supplemented with increasing concentrations of nickel or cobalt chloride, and disk diffusion tests of these metals revealed a large diversity of resistance to nickel and cobalt (Table 1). Strain UPM1137 showing the minimum diameter of the inhibition zone in the disk diffusion tests, and strain UPM1136 showing the maximum value, were selected as the most and less resistant strains, respectively.

Table 1. Susceptibility of *R. leguminosarum* bv. viciae strains to transition metal ions as determined by disk diffusion tests.

Strains	Inhibition zone diameter (mm)	
	$NiCl_2$*	$CoCl_2$+
UPM1131[2]	20	20
UPM1132[2]	18	26
UPM1133[2]	16	18
UPM1134[2]	18	16
UPM1135[1]	16	22
UPM1136[2]	26	26
UPM1137[2]	12	15
UPM1138[2]	22	26
UPM1139[2]	14	24
UPM1140[1]	17	18
UPM1141[1]	22	20
UPM1142[1]	22	15
3841	20	20

[1] Isolated from contaminated soil (Stuttgart, Germany).
[2] Isolated from ultramafic soil (Gorro, Italy).
*200 mM $NiCl_2$. +100 mM $CoCl_2$

In order to identify genetic systems involved in nickel and cobalt resistance, a random mutagenesis was carried out in strain UPM1137 by the insertion of a Tn5-derivative minitransposon. The screening for nickel resistance revealed that 39 out of 4313 transconjugants were unable to grow at the nickel concentration established (1.5 mM $NiCl_2$). So far, the disrupted genes have been identified in 26 mutants, and the comparison of the translated sequences enabled protein classification in functional categories. The most frequent category corresponded to secreted and membrane proteins, 26%, followed by proteins involved in the metabolism of small molecules, 23%. Proteins identified in this analysis included a homolog to *E. coli* Ni/Co efflux system RcnA, and a putative transmembrane protein found in other *Rhizobiaceae* species and encoded in a locus adjacent to the copper homeostasis *cop* operon. The characterization of nickel and cobalt resistance levels of the mutants established two different groups, one of them consisting of mutants only affected in nickel resistance and the other being sensitive to both metals. Further phenotypic characterization of these mutants will determine the role of the genetic systems identified in metal homeostasis.

References

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